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## The Peroxidase-like Activity of the Hemoglobin-Haptoglobin Complex<sup>1)</sup>

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There is a conflict in the literature as to whether haptoglobin enhances or inhibits the peroxidase like activity of hemoglobin. This conflict arises from: 1. the effect of the concentration of  $H_2O_2$ <sup>4)</sup> on the activity, 2. the use of different parameters to express the activity.

In our own experience using *o*-tolidine as the donor, we found that, depending on the concentration of  $H_2O_2$ , activation or inhibition by haptoglobin was observed when the maximum color development was the parameter for the activity. With low concentrations of  $H_2O_2$  activation and with higher concentrations of  $H_2O_2$  inhibition was found. However, when the initial reaction rate was used to express the activity, there always was activation regardless of the peroxide concentration.

The sequence of events when  $H_2O_2$  is added to  $HgbO_2$  and  $HgbO_2$  : Hp and Methgb and Methgb : Hp was followed by observing changes in the spectra. The results from the change of the spectra together with the reaction rate of curves of the peroxidase reactions lead us to the conclusion that the complex formed between hemoglobin and haptoglobin enhances the peroxidase like activity of hemoglobin and that it also increases the susceptibility to the destruction by  $H_2O_2$ .

Widersprechende Resultate in der Literatur darüber, ob Haptoglobin die peroxydaseähnliche Aktivität von Hämoglobin beschleunigt oder hemmt, lassen sich auf zwei Ursachen zurückführen: 1. den Effekt der Konzentration von  $H_2O_2$  auf die Enzymaktivität und 2. den Gebrauch verschiedener Parameter für die Enzymaktivität.

Wir fanden, daß mit *o*-Tolidin als Chromogen und der maximalen Farbentwicklung als Maß für die Aktivität, Aktivierung oder Hemmung durch Haptoglobin von der  $H_2O_2$ -Konzentration abhängt. Bei niedrigen  $H_2O_2$ -Konzentrationen beobachteten wir Aktivierung, bei höheren Hemmung. Wird die Enzymaktivität jedoch auf die Anfangsgeschwindigkeit der Reaktion bezogen, so fanden wir bei allen  $H_2O_2$ -Konzentrationen eine Beschleunigung.

Der Reaktionsablauf bei der Einwirkung von  $H_2O_2$  auf  $HgbO_2$ ,  $HgbO_2$  : Hp, Methgb und Methgb : Hp wurde mit Registrierung der Spektren verfolgt. Diese Resultate, zusammen mit dem Verlauf der Zeit-Umsatzkurven der Enzymreaktion, deuten wir folgendermaßen: der Hämoglobin : Haptoglobin-Komplex hat eine höhere Peroxydaseaktivität als freies Hämoglobin, ist aber gleichzeitig einer schnelleren Zerstörung durch  $H_2O_2$  ausgesetzt.

In a preceding paper (1) we discussed the difficulties one encounters in the determination of microgram-quantities of hemoglobin in urine. Urine contains potent inhibitors for the peroxidase-like activity of hemoglobin. By gel filtration with Sephadex hemoglobin can be separated from the inhibitors. Recovery of hemoglobin from Sephadex columns and separation from the inhibitors were greatly increased when hemoglobin was present as the hemoglobin : haptoglobin complex. When haptoglobin was added to a hemoglobin solution, we observed a sharp decrease of the peroxidase-like activity (max.

O. D.) under the conditions of our peroxidase-assay-system, which contained *o*-tolidine as chromogen and 640 mM  $H_2O_2$ .

This observation was in contrast to the report by CONNELL and SMITHIES (2) who found that Hp enhances the oxidation of guaiacol by Hgb in the presence of  $H_2O_2$ . Initially we believed that this difference was based on the unlikeness of the chromogens - guaiacol and *o*-tolidine. Our belief was supported by the results from LUPOVITCH and ZACK (3), who found inhibition of the peroxidase-like activity of Hgb by Hp with *o*-dianisidine (like *o*-tolidine a derivate of benzidine) as chromogen.

Only when we altered the assay conditions in our system, in order to study the initial velocity of the peroxidase reaction, did we observe that the activity (max. O. D.) of the Hgb : Hp-complex with one and the same chromogen can be higher or lower than the activity of free Hgb, depending *inter alia* on the concentration of  $H_2O_2$ . Recently TARUKOSKI (4) reported activation of peroxidase activity of Hgb by Hp, with *o*-dianisidine and ethylhydroperoxide.

The present report describes the effect of Hp and  $H_2O_2$  on the peroxidase-like activity of Hgb.

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<sup>4)</sup> Abbreviations used in this work: EtOOH: Ethylhydroperoxide; Hgb: hemoglobin; Hp: haptoglobin;  $H_2O_2$ : hydrogen peroxide; Methgb: methemoglobin; Methgb-OOH: Methemoglobinhydroperoxide complex;  $HgbO_2$ : Oxyhemoglobin; Hgb (hemoglobin) is used synonymously with  $HgbO_2$  (oxyhemoglobin). Only in the spectra is the abbreviation  $HgbO_2$  to avoid misunderstanding.

## Methods

### Reagents

**Hemoglobin (Hgb):** Hgb was prepared by lysing human erythrocytes with distilled  $H_2O$  and standardized as cyanmethemoglobin. A Hgb stock solution with appr. 60 mg/ml was stable for at least four weeks when kept refrigerated. Each day appropriate dilutions were prepared by dilution with saline.

**Haptoglobin (Hp):** Human Hp was prepared for us by Drs. NIRSCHMANN and STAUFFER (5), University of Berne, Switzerland. The preparation used in the presented study contained appr. 50% Hp and was free of Hgb. Solutions were prepared in saline.

**Hemoglobin-Haptoglobin-Complex (Hgb:Hp):** equal volumes of Hgb and Hp were mixed. Ratio Hgb/Hp at least 1:1 (calculated as pure Hp).

**o-Tolidine-tartrate-buffer (reagent buffer):** 43.6 mg o-tolidine-2HCl were dissolved in 100 ml 0.1M tartrate buffer pH 4.2 (correction of pH to 4.2 if necessary). For some experiments o-dianisidine was used. (o-Dianisidine-2HCl was dissolved in hot water, filtered over charcoal and recrystallized).

**Hydrogenperoxide:** fresh dilutions were prepared each day from a 30% solution (standardized with permanganate titration).

### Peroxidase test

The maximum absorbance of the blue reaction product with o-tolidine is at 635 nm. Some experiments were done at this wavelength in a Zeiss PMQ II spectrophotometer, others were measured in an Eppendorf photometer with filter Hg 578 nm (mercury light source). Although the absorbance of the blue product is less at 578 nm by the factor of appr. 1.8, measurements with the Eppendorf photometer were preferred because of certain technical advantages of this instrument. All measurements were performed at 25°C in a thermostat cuvette holder. The maximum absorbance of the green reaction product with o-dianisidine is at 690 nm. Measurements were made at this wavelength in the Zeiss spectrophotometer and reaction curves were recorded. Reagent buffer 2.25 ml and 900  $\mu$ l saline were mixed in a 10 mm cuvette; 100  $\mu$ l Hgb or Hgb:Hp were added by pipetting the solution into the reagent mixture without touching the wall of the cuvette! After mixing the O. D. was adjusted to zero. The reaction was started by adding 250  $\mu$ l  $H_2O_2$  under vigorous stirring with a motor driven rotating mixer.

**Final concentrations:** o-Tolidine or o-dianisidine 1 mM,  $H_2O_2$  varied in most experiments. Total volume of digest: 3.5 ml. Polyethylene constriction pipettes (6) were used for Hgb, Hgb:Hp and  $H_2O_2$ .

**Parameters for the peroxidase activity:** The "activity" has to be more clearly defined. Correctly the parameter should be the initial velocity of the enzyme reaction. But some investigators, including ourselves (1), have chosen the maximum of the color development as parameter. It is our experience that the initial velocity ( $\Delta$  O. D./min.) at least in our system can not be measured with the same accuracy as max. O. D., in other words, the same max. O. D. can be reached in repeatedly performed and identical experiments, but the  $\Delta$  O. D. can vary considerably.

This point becomes important when the amount of Hgb is to be measured. The most likely reason for the poorer reproducibility of  $\Delta$  O. D./min. will be discussed below. In the following discussion we shall in all instances denote whether the initial velocity or the maximum color development has been used to express the peroxidase activity.

### Spectra of Hgb:Hp and their complex with $H_2O_2$

Spectra of Hgb (oxyhemoglobin), Hgb:Hp, Methgb and Methgb:Hp were recorded automatically between 500 and 650 nm in a Unicam SP 800. The various hemoglobins were added to tartrate buffer pH 4.2, the final concentration of Hgb was 0.65 mg/ml or  $39 \times 10^{-6}$  M (calculated per heme).  $H_2O_2$  was added in a molar ratio "R" =  $\frac{[H_2O_2]}{[heme]}$ . The time for recording the spectrum was

40 sec. In a second series of experiments the spectra were recorded by handoperation in a Zeiss PMQ II spectrophotometer equipped with a microliter attachment. To save the valuable Hp the volumes were reduced to a total of 200  $\mu$ l. At various times suitable aliquots were withdrawn (mostly 10  $\mu$ l) and immediately added to the standard peroxidase test mixture with o-tolidine, and the peroxidase activity was measured in the presence of 64 mM  $H_2O_2$ .

## Results

### Variation of $H_2O_2$ Concentration

#### Hgb and Hgb:Hp with o-tolidine as chromogen

The concentration of  $H_2O_2$  was varied from 10–500 mM (fig. 1) and from 0.85–34 mM (fig. 1a). The activity (max. O. D.) of Hgb increased with increasing concentrations of  $H_2O_2$  and reached a plateau at about 200 mM  $H_2O_2$ . Only at concentrations above 500 mM  $H_2O_2$  did the activity slightly decline. The initial velocity ( $\Delta$  O. D./min.) with Hgb increased with the  $H_2O_2$  concentration; the curve rose rather sharply at concentrations of  $H_2O_2$  up to about 40 mM, to become less steep and directly related to the concentration of  $H_2O_2$ .

The activity (max. O. D.) of Hgb:Hp increased slightly with decreasing concentrations of  $H_2O_2$  between 500 and 250 mM. With lower concentrations of  $H_2O_2$  the increase became exponential (fig. 1) and reached a peak at approximately 4–5 mM  $H_2O_2$ . At a lower  $H_2O_2$  concentration the activity declined sharply. The initial velocity ( $\Delta$  O. D./min.) at all levels of  $H_2O_2$  was very much higher than with free Hgb. The curve for Hgb:Hp was almost parallel to the curve with Hgb. The max. O. D. with Hgb:Hp was lower than with Hgb at high concentrations of  $H_2O_2$  and higher with low concentrations of  $H_2O_2$ . At about 90 mM the two curves crossed (fig. 1) which means that the reaction catalyzed by free Hgb and Hgb:Hp reached the same max. O. D. Figure 3 shows the time-reaction curves for Hgb and Hgb:Hp with three concentrations of  $H_2O_2$ . Hp accelerated the initial velocity at all levels of  $H_2O_2$ ; but the curves bent sharply at high concentrations, while the lower the concentrations of  $H_2O_2$ , the more of the blue oxidation product of o-tolidine was formed. Free Hgb behaved conversely. Initial velocity and maximum color formation increased with the concentration of  $H_2O_2$ . At 90 mM  $H_2O_2$  the reactions with Hgb and Hgb:Hp reached about the same max. O. D., but the initial velocity ( $\Delta$  O. D./min.) of the reaction with Hgb:Hp was faster than that with free Hgb.

#### Hgb and Hgb:Hp with o-dianisidine as chromogen

Generally the same types of curves were obtained with o-dianisidine as with o-tolidine. Equal max. O. D. were obtained with Hgb and Hgb:Hp at about 130 mM  $H_2O_2$  (fig. 2). The time reactions curves (fig. 4) also demonstrated the accelerating effect of Hp on the initial velocity of the reaction at all levels of  $H_2O_2$ . The green oxidation product of o-dianisidine was less stable than the blue oxidation product of o-tolidine and hence the curves declined at a faster rate.

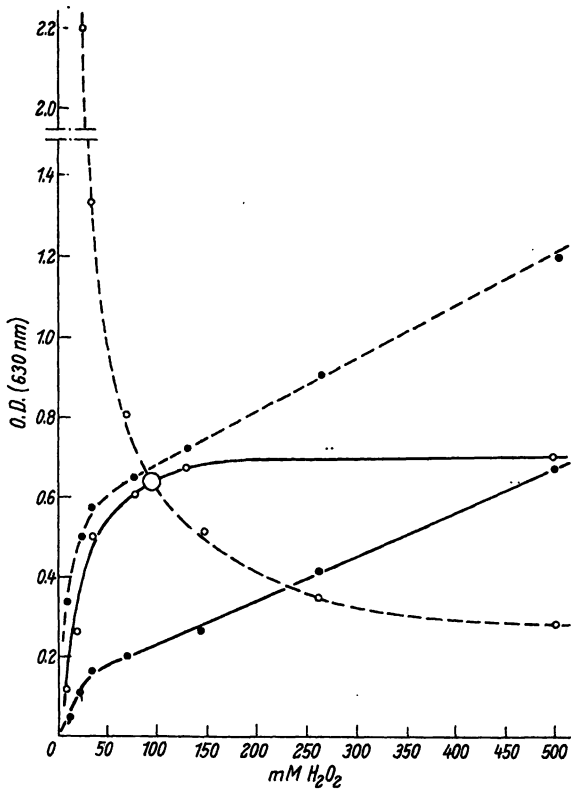


Fig. 1

Variation of  $H_2O_2$ , 15—500 mM. Chromogen: o-tolidine. Hgb = 7  $\mu$ g, Hp-preparation = 14  $\mu$ g.

Peroxidase activity:  
Hgb, max. O. D. ○—○,  $\Delta$  O. D./min. ○—○  
Hgb: Hp, max. O. D. ○—○,  $\Delta$  O. D./min. ○—○

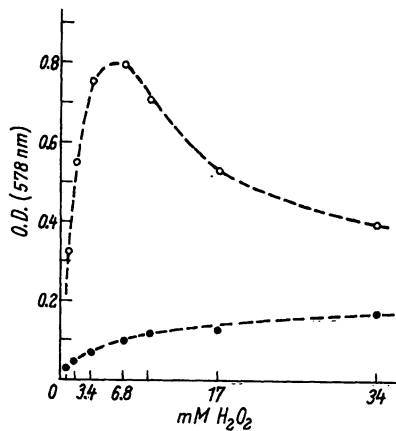


Fig. 1a

Variation of  $H_2O_2$ , 85—34 mM. Chromogen: o-tolidine. Hgb = 3.8  $\mu$ g, Hp-preparation = 8  $\mu$ g.

Peroxidase activity:  
Hgb: Hp ○—○ max. O. D., ○—○  $\Delta$  O. D./min.

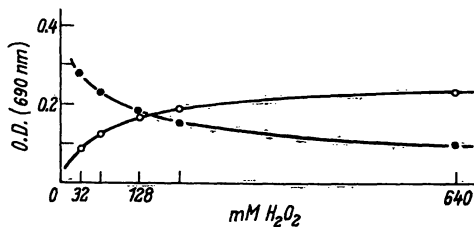


Fig. 2

Variation of  $H_2O_2$ , 32—640 mM. Chromogen: o-dianisidine. Hgb = 7  $\mu$ g, Hp-preparation = 14  $\mu$ g. Tartrate buffer pH 4.2.

Peroxidase activity:  
Hgb ○—○, max. O. D.,  
Hgb: Hp ○—○, max. O. D.

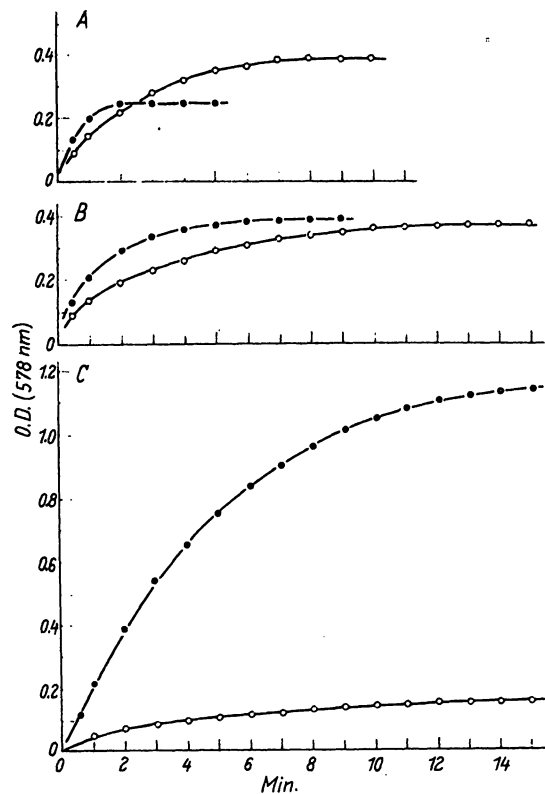


Fig. 3

Time reaction curves with o-tolidine at various concentrations of  $H_2O_2$ :

A = 128 mM, B = 90 mM, C = 16 mM. Hgb = 6  $\mu$ g, Hp-preparation = 12  $\mu$ g.  
Hgb ○—○, Hgb: Hp ○—○

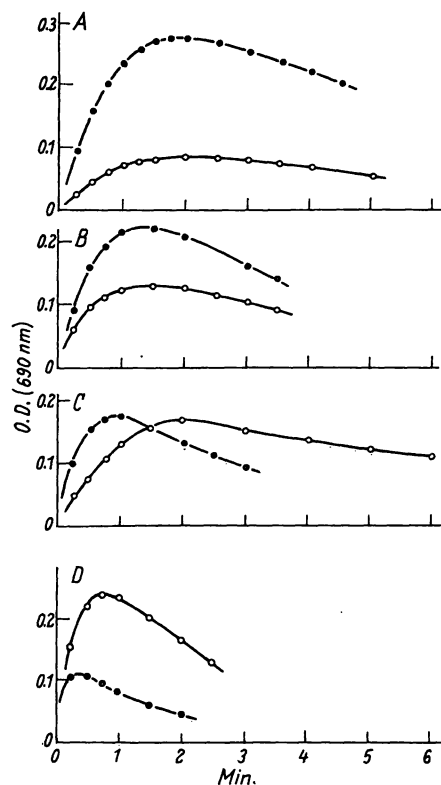


Fig. 4

Time reaction curves with o-tolidine (tartrate buffer pH 4.2) at various concentrations of  $H_2O_2$ :

A = 32 mM, B = 64 mM, C = 128 mM, D = 640 mM. Hgb = 7  $\mu$ g, Hp-preparation = 14  $\mu$ g.  
Hgb ○—○, Hgb: Hp ○—○

## Variation of o-tolidine

The effect of the concentration of o-tolidine was measured at three concentrations of  $H_2O_2$  — 64, 213 and

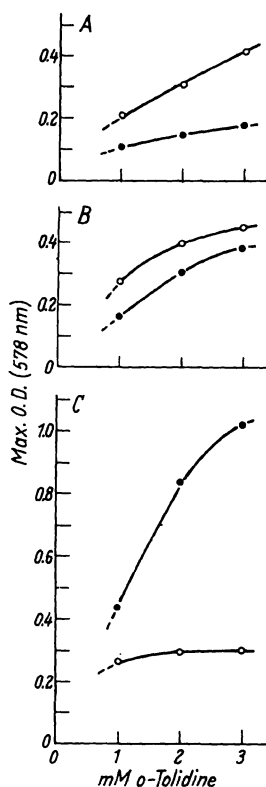


Fig. 5  
Variation of o-tolidine at various concentrations of  $H_2O_2$ :  
A = 640 mM, B = 213 mM,  
C = 64 mM.  
Hgb = 7  $\mu$ g, Hp-preparation = 14  $\mu$ g.  
Hgb =  $\circ$ — $\circ$ , Hgb:Hp =  $\bullet$ — $\bullet$ .

640 mM (see fig. 5). At all concentrations of  $H_2O_2$  we found an increase of the activity (max. O. D.) of both Hgb and Hgb:Hp. With 64 mM  $H_2O_2$  the effect of concentration of o-tolidine on Hgb activity was only very slight, but the activity of Hgb:Hp increased sharply with concentration of o-tolidine. Due to the poor solubility of o-tolidine the use of final concentrations of o-tolidine greater than 1 mM is impractical.

The effect of the sequence of addition of the components of the reaction on the peroxidase activity (all experiments with 64 mM  $H_2O_2$ )

The sequence in which the Hgb-solution and the reagents were pipetted into the cuvette was of influence on the peroxidase activity. Whenever a Hgb-solution was added to the cuvette first, a very low activity was obtained. It was observed that Hgb sticks to the glass and forms there a blue precipitate as soon as the reaction has been started. Adhering to the glass was occasionally also observed when Hgb was pipetted into a solution in the cuvette by holding the tip of the pipette to the glass wall. Thereby, the activity was decreased. When Hgb-solutions are to be pipetted into another solution, touching the glass wall with the tip of the pipette should be avoided. When  $H_2O_2$  was added to Hgb or Hgb:Hp prior to the chromogen, no color development occurred.

A standard procedure was adopted to pipette the solutions in the following order: chromogen-buffer, saline, Hgb,  $H_2O_2$ . When Hgb was added last, max. O. D. was only slightly higher, but the initial velocity ( $\Delta$  O. D./

min.) was found to be almost double (tab. 1). This led to investigating the influence of the time Hgb was exposed to the o-tolidine-buffer, before the reaction was started with  $H_2O_2$  (tab. 2).

Tab. 1  
Variation of the sequence of adding o-tolidine (T), Hgb, saline (S) and  $H_2O_2$ . Hgb = 7  $\mu$ g,  $H_2O_2$  = 64 mM

Sequence	max. O. D. $\times 10^3$	$\Delta$ O. D. $\times 10^3$ /min.
T—Hgb—S— $H_2O_2$	300	180
T—S—Hgb— $H_2O_2$	300	180
T—S— $H_2O_2$ —Hgb	325	350

Tab. 2  
Variation of time before adding  $H_2O_2$  to the mixture of o-tolidine (T), saline (S) and Hgb. Hgb = 7  $\mu$ g,  $H_2O_2$  = 64 mM

Time before adding $H_2O_2$ to T—S—Hgb	max. O. D. $\times 10^3$	Time [min.] to reach max.	$\Delta$ O. D. $\times 10^3$ /min.
20 sec.	300	4	340
1 min.	300	4	240
4 min.	300	9	125
7 min.	280	10	125
13 min.	280	10	125

From 20 sec. to 4 min. identical max. O. D. was found and even after 13 min. this value decreased only slightly. But a marked difference was observed in the initial velocity, which declined with time. This means that the initial velocity of the reaction has no relation to the maximum color development. With Hgb:Hp the sequence of addition had no significant influence on max. O. D., whether or not the solutions of Hgb and Hp were premixed or added separately (tab. 3). A definitely lower max. O. D. was found when Hgb:Hp was added last.  $\Delta$  O. D./min. varied somewhat but in no obvious relation to the sequence of addition.

Tab. 3  
Variation of the sequence of adding Hgb, Hp, o-tolidine (T), saline (S), and  $H_2O_2$ . Hgb = 7  $\mu$ g, Hp-preparation = 14  $\mu$ g,  $H_2O_2$  = 64 mM

Sequence	max. O. D. $\times 10^3$	$\Delta$ O. D. $\times 10^3$ /min.
T—S—Hgb— $H_2O_2$ (control)	300	220
T—S—Hp— $H_2O_2$ (control)	0	—
S—T—(Hgb:Hp)— $H_2O_2$	420	275
T—S—(Hgb:Hp)— $H_2O_2$	430	220
T—S— $H_2O_2$ —(Hgb:Hp)	375	240
T—Hgb—Hp—S— $H_2O_2$	420	250
Hp—T—Hgb—S— $H_2O_2$	430	240
S—Hgb—Hp—T— $H_2O_2$	440	260

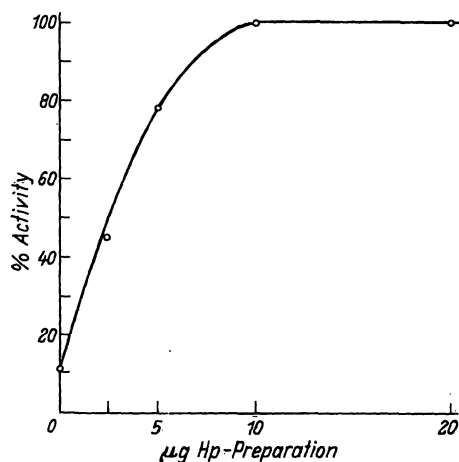


Fig. 6  
Variation of the amount of Hp. Concentration of  $H_2O_2$  = 20 mM.  
Hgb = 5  $\mu$ g

Tab. 5  
Relative peroxidase activities of HgbO<sub>2</sub> and Methgb, and their complexes with Hp, measured concomitantly with their spectra without and in the presence of H<sub>2</sub>O<sub>2</sub>

R <sup>1)</sup>	time <sup>2)</sup> (min.)	HgbO <sub>2</sub>			time (min.)	HgbO <sub>2</sub> : Hp		
		$\Delta$ O. D. min. (%)	max. O. D. (%)	spectrum <sup>3)</sup> Nr.		$\Delta$ O. D. min. (%)	max. O. D. (%)	spectrum Nr.
0	1	100	100	I	1	100	100	IV
500	3	19	53	II	2	29	38	—
500	15	17	50	III	12	24	38	V
0	1	100	100	VI	1	100	100	X
500	2	6	20	VII	1	2	9	XI
5	1	100	100	VIII	1	74	86	XII
5	12	44	96	IX	12	64	49	XIII

<sup>1)</sup>  $R = \frac{[H_2O_2]}{[heme]}$

<sup>2)</sup> Time of incubation at various R-values before aliquot was withdrawn for peroxidase activity measurement.

<sup>3)</sup> Curves I—III see fig. 11, IV and V fig. 12, VI—IX fig. 13, X—XIII fig. 14.

### The saturation of Hgb with Hp

The molecular weight of Hp is approximately 85,000 and Hp binds to Hgb in a molar ratio of 1 : 1 (8). 1  $\mu$ g Hgb binds accordingly 1.25  $\mu$ g Hp. The Hp-preparation of NITSCHMANN und STAUFFER (5) was claimed by the authors to contain at least 50% Hp. This is expressed as Hp and not Hgb combining equivalent as other authors have used.

Our results confirm this activity. With H<sub>2</sub>O<sub>2</sub> concentrations < 90 mM the peroxidase activity of Hgb (max. O. D. and  $\Delta$  O. D./min.) increases until saturation is reached. At higher concentrations of H<sub>2</sub>O<sub>2</sub> max. O. D. decreases until the saturation point is reached (1).

### Spectra of Hgb and Hgb:Hp and their complexes with H<sub>2</sub>O<sub>2</sub>

The results are given in figures 8—14 and table 5. For the interpretation of the curves see the discussion.

### Concentration of Hgb and Hgb:Hp versus peroxidase activity at various levels of H<sub>2</sub>O<sub>2</sub> concentrations

A directly proportional relationship between the concentration of Hgb and the peroxidase activity was found at the investigated concentrations of H<sub>2</sub>O<sub>2</sub> between 32 and 128 mM and as reported earlier at 640 mM (1). The relationship deviated from linearity with Hgb:Hp at H<sub>2</sub>O<sub>2</sub> concentrations less than 128 mM.

At lower concentrations the peroxidase activity was unproportionately higher with larger amounts of Hgb:Hp (fig. 16).

### Discussion

JAYLE (7) observed that Hp accelerated the Hgb catalyzed oxidation of iodide by ethylhydroperoxide, and developed a method for the determination of Hp based on this principle. NYMAN (8) reinvestigated and extended this study and confirmed the activating effect of Hp. CONNELL and SMITHIES (2) described the same effect of Hp. At pH 4 free Hgb had almost no peroxidase activity with guaiacol as chromogen and H<sub>2</sub>O<sub>2</sub>, while the Hgb:Hp complex was active.

In contrast, LUPOVITCH and ZACK (3) reported that with o-dianisidine and H<sub>2</sub>O<sub>2</sub> the activity of Hgb:Hp was less

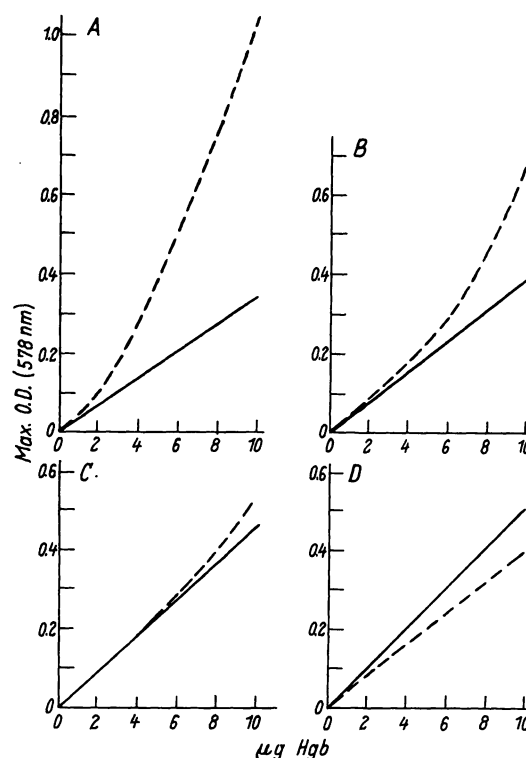


Fig. 16

Concentration of Hgb (—) and Hgb:Hp (---) versus peroxidase activity at various concentrations of H<sub>2</sub>O<sub>2</sub>;

A = 32 mM, B = 64 mM, C = 90 mM, D = 128 mM

than the activity (max. O. D.) of free Hgb. These authors quoted NYMAN (8) incorrectly in stating that NYMAN found an inhibitory effect of Hp. TARUKOSKI (4) used o-dianisidine and Ethylhydroperoxide and found Hgb:Hp to be more active (max. O. D.) than free Hgb. The differences in the experimental conditions used by the two groups of investigators were the following: LUPOVITCH and ZACK employed H<sub>2</sub>O<sub>2</sub> at 0° C and TARUKOSKI Ethylhydroperoxide at room temperature. Our investigations demonstrated that the effect of Hp depended inter alia on the concentration of H<sub>2</sub>O<sub>2</sub>. Figure 1 shows two pairs of curves: the activity of free Hgb and of Hgb:Hp expressed as  $\Delta$  O. D./min. and a max. O. D. Expressed as  $\Delta$  O. D./min. Hp activated the peroxidase activity at all levels of H<sub>2</sub>O<sub>2</sub>, and the activity of both free Hgb and Hgb:Hp increased with in-

creasing concentrations of  $H_2O_2$ . In contrast the curves for the maximum color development (max. O. D.) were entirely different.

With free Hgb max. O. D. increased with increasing amounts of  $H_2O_2$  until a plateau was reached at about 200 mM. In the presence of Hp the max. O. D. increased with decreasing concentrations of  $H_2O_2$  at first slightly and from about 100 mM  $H_2O_2$  down almost exponentially (fig. 1). The curve reached a peak at approx. 4–5 mM  $H_2O_2$  and then declined (fig. 1a). The curves for Hgb : Hp and Hgb crossed at about 90 mM  $H_2O_2$ . Results following the same pattern were obtained with o-dianisidine as chromogen in tartrate buffer (fig. 2 and 4).

LUPOVITCH and ZACK (3) found inhibition of the peroxidase-like activity of free hemoglobin by Hp with o-dianisidine as chromogen at a level of  $H_2O_2$  concentration, where we found activation (64 mM  $H_2O_2$ , see fig. 2). Their test conditions varied from ours as follows: o-dianisidine in acetate buffer, pH 3.8 and incubation temperature  $0^\circ C$ . We repeated the procedure accordingly incubating at  $25^\circ$  and  $0^\circ C$ . At  $25^\circ C$  the activity of Hgb : Hp increased slightly with decreasing  $H_2O_2$  concentration, and the activity of Hgb increased sharply with increasing

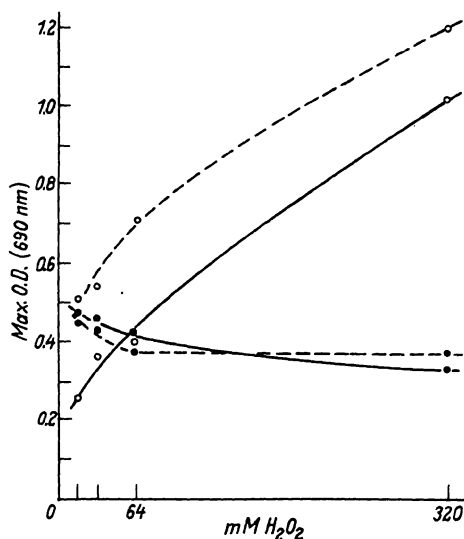


Fig. 7

Variation of  $H_2O_2$ , 16–320 mM. Chromogen: o-dianisidine. Acetate buffer pH 3.8 (according to LUPOVITCH and ZACK (3)). Incubation at  $0^\circ C$  (— — —) and at  $25^\circ$  (—). Hgb o, Hgb : Hp •. Hgb = 7  $\mu g$ , Hp-preparation = 14  $\mu g$

$H_2O_2$  concentration. The two curves crossed at about 64 mM  $H_2O_2$  (fig. 7). It must be emphasized that the conditions for the experiment in figure 7 differed from those in figure 2, where tartrate buffer of pH 4.2 was used. Whether the difference in pH or the nature of the buffer caused the shift of the crossing point of the Hgb and Hgb : Hp curves from about 170 mM  $H_2O_2$  to about 64 mM  $H_2O_2$  has not been investigated. At  $0^\circ C$  the two curves do not cross in the range between 16 and 320 mM  $H_2O_2$ , but at 16 mM  $H_2O_2$  the activity of Hgb : Hp was only 10% less than that of free Hgb. At 64 mM  $H_2O_2$  the activity of Hgb : Hp was about 50% of that of Hgb, which agrees well with the results of LUPOVITCH and ZACK. The authors described a photochemical effect, that is an enhancement of the reaction with o-tolidine, by white light and almost complete inhibition of color development in the dark.

We were not able to confirm this. In our experiments there was no difference in max. O. D. or the shape of the reaction curves with free Hgb and Hgb : Hp whether we incubated in the light or in the dark. It should be mentioned that we used o-dianisidine-HCl which was filtered over charcoal and recrystallized. Maybe impurities in the chromogen caused the effect observed by LUPOVITCH and ZACK.

Table 4 summarizes the results cited from the literature in comparison to our investigation. We had found earlier (1) that the relationship between the concentration of Hgb and of Hgb : Hp and the peroxidase-like activity was directly proportional. While this held true for Hgb at  $H_2O_2$  concentrations investigated down to 32 mM, the relationship deviated from linearity with Hgb : Hp at  $H_2O_2$  concentrations below 128 mM (fig. 16). We are at present not able to give any explanation for the latter fact, but it becomes obvious that any quantitative determinations of the Hgb : Hp complex with o-tolidine and  $H_2O_2$  concentrations smaller than 128 mM require calibration curves.

KEILIN and HARTREE (9) observed spectroscopically the formation of a complex between Methgb and  $H_2O_2$ , similar to the horseradish peroxidase-hydroperoxide complex III with characteristic bands ( $\alpha$ ) 589 and ( $\beta$ ) 545 nm in solutions between pH 5.5 and 9.2. When the molar ratio (R) of  $H_2O_2$  to heme was  $R = 1$  the formation of Methgb-OOH was 80% complete. Between  $R = 2.5$ –5 the complex formation was 100%, but on

Tab. 4

The effect of Hp on the peroxidase activity (max. O. D.) of Hgb. Comparison of test conditions

Author	Electron donor	Peroxide	Peroxide Concentrations mM	Buffer pH	Temp. $^\circ C$	Effect of Hp	Figure Nr.
CONNELL et al. (2)	Guaiacol	$H_2O_2$	< 10–10	Acetate 4.2	$30^\circ$	Activation	
Present study	Guaiacol	$H_2O_2$	< 10–250	Acetate 4.2	$25^\circ$	Activation	
NYMAN (8)	Iodide	EtOOH	7	Acetate 4.4	$32^\circ$	Activation	
TARUKOSKI (4)	o-Dianisidine	EtOOH	3	Phosphate 4.1	room	Activation	
LUPOVITCH (3) et al.	o-Dianisidine	$H_2O_2$	64	Acetate 3.8	$0^\circ$	Inhibition	
Present study	o-Dianisidine	$H_2O_2$	16	Acetate 3.8	$0^\circ$	Inhibition	7
Present study	o-Dianisidine	$H_2O_2$	64	Acetate 3.8	$25^\circ$	Inhibition	7
Present study	o-Dianisidine	$H_2O_2$	64	Acetate 3.8	$25^\circ$	Activation	7
Present study	o-Dianisidine	$H_2O_2$	130	Tartrate 4.2	$25^\circ$	Inhibition	2
Present study	o-Dianisidine	$H_2O_2$	130	Tartrate 4.2	$25^\circ$	Activation	2
Present study	o-Tolidine	$H_2O_2$	90	Tartrate 4.2	$25^\circ$	Inhibition	1
Present study	o-Tolidine	$H_2O_2$	90	Tartrate 4.2	$25^\circ$	Activation	1

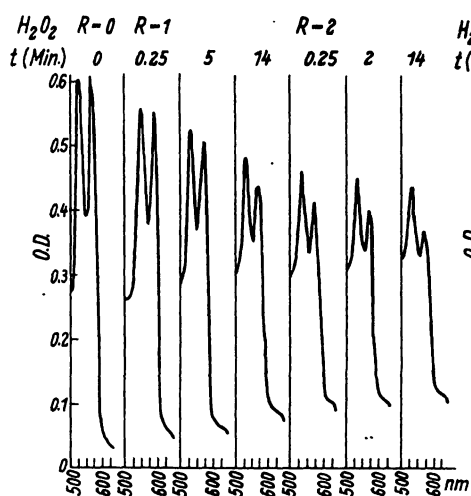


Fig. 8

Spectra of  $\text{HgbO}_2$  without and with  $\text{H}_2\text{O}_2$ , tartrate buffer pH 4.2. Changes with time after the addition of  $\text{H}_2\text{O}_2$ .

$R = \frac{[\text{H}_2\text{O}_2]}{[\text{heme}]} \cdot \text{Heme} = 45 \mu\text{M}$ . For details see text. The curves were treated from recorded curves

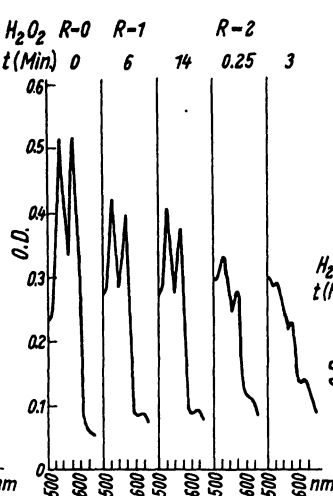


Fig. 9

Spectra of  $\text{HgbO}_2$ : Hp without and with  $\text{H}_2\text{O}_2$ , tartrate buffer pH 4.2.

Heme =  $39 \mu\text{M}$ . See legend to fig. 8

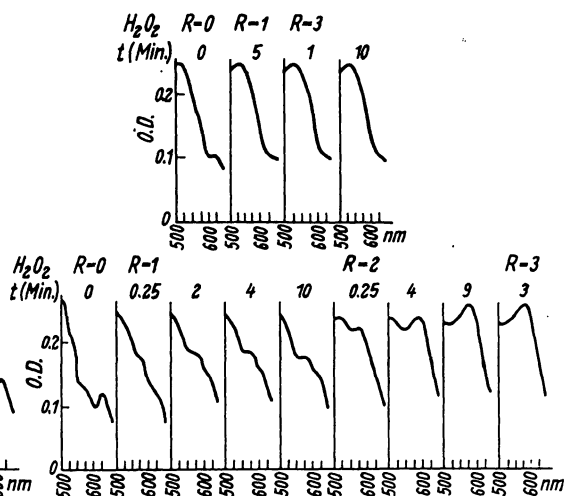


Fig. 10

Spectra of Methgb (upper curves) and Methgb: Hp (lower curves) without and with  $\text{H}_2\text{O}_2$ , tartrate buffer pH 4.2.

Heme =  $23.2 \mu\text{M}$

standing Methgb was liberated and underwent destruction. At  $R = 25$  the band split into two peaks at 578 and 592 nm. With an even higher concentration of  $\text{H}_2\text{O}_2$  ( $R = 250$ – $500$ ) the band at 592 nm became invisible and the spectrum was indistinguishable from that of  $\text{HgbO}_2$ .

From this the authors concluded that the reaction between Methgb and  $\text{H}_2\text{O}_2$  is as follows: formation of Methgb-OOH which on further reaction with  $\text{H}_2\text{O}_2$  decomposes  $\text{H}_2\text{O}_2$  to  $\text{O}_2$  and  $\text{H}_2\text{O}$ . This reaction is accompanied by the reduction of Methgb to Hgb and the oxygenation of the latter to  $\text{HgbO}_2$ . The decomposition of  $\text{H}_2\text{O}_2$  and the appearance of  $\text{HgbO}_2$  are followed by the destruction of the heme nucleus, with disappearance of the absorption bands.

At pH 4.5 KEILIN and HARTREE (9) found that with  $R = 100$  Methgb was gradually replaced by a relatively stable compound with a wide absorption band 590 nm. There was evidence that oxidation of the porphyrin ring occurred, but the authors did not mention whether they regard this compound as catalytically inactive. With a great excess on  $\text{H}_2\text{O}_2$   $R=100$ , Methgb was rapidly replaced by  $\text{HgbO}_2$ .

We recorded the spectra of Methgb and  $\text{HgbO}_2$  with and without Hp in the absence of and in the presence of various concentrations of  $\text{H}_2\text{O}_2$  in tartrate buffer pH 4.2 (conditions of our assay system). Two sets of experiments were conducted. From the results in the first series we obtained information on the time course of the changes of the spectra in dependence on  $R$ . With  $R = 1$  the spectrum of  $\text{HgbO}_2$  (fig. 8) changed gradually. The peaks at 540 and 575 nm decreased. When  $R$  was increased after 14 min. to 2 the change was a little faster. The same experiment was repeated with  $\text{HgbO}_2$ -Hp (fig. 9), the changes occurred faster.

Methgb (fig. 10) had a broad maximum between 500 and

530 nm and a lower peak at 625 nm, and hinted peaks at 545 and 580 nm. Immediately after the addition of  $\text{H}_2\text{O}_2$ ,  $R = 1$ , the peak at 625 nm diminished, and there was very little further change within the next 5 min. At that time  $R$  was increased to 3 and no further change was observed during the next 10 min.

The spectrum of Methgb: Hp was different (fig. 10) with a maximum and peak at 500 nm and shoulder between 560 and 580 nm and a distinct peak at 630 nm. Immediately after the addition of  $\text{H}_2\text{O}_2$ ,  $R = 1$ , the spectrum began to change, and over the next 4 min. the peak at 500 decreased slightly and a peak at 580 nm became distinct, while the peak at 630 nm diminished. No further change was observed during the following 6 min., at which time  $\text{H}_2\text{O}_2$  was increased to  $R = 2$ . The peak at 500 nm decreased further and the peak at 580 nm increased and shifted to 590 nm. On increasing  $R$  to 3 no further change occurred.

In the second series the spectra were recorded concomitant with activity measurements (tab. 5). The change of the spectrum of  $\text{HgbO}_2$  after addition of  $\text{H}_2\text{O}_2$ ,  $R = 500$ , is shown in figure 11. The curves approached the spectrum of Methgb, but the peaks at 540 and 575 nm were still prominent. The activity after 3 min. (curve II) was 53% of that before the addition of  $\text{H}_2\text{O}_2$  and after 15 min. (curve III) was still 50%. This means that about 50% of the Hgb must have been "destroyed", because Hgb and Methgb have the same peroxidase activity.

The spectrum of  $\text{HgbO}_2$ : Hp (fig. 12, curve IV) was identical with that of  $\text{HgbO}_2$ . Twelve minutes after the addition of  $\text{H}_2\text{O}_2$ ,  $R = 500$ , a change similar to that with Hgb was seen in curve V, but the activity was only 38% of that before the addition of  $\text{H}_2\text{O}_2$ . In the presence of Hp the "destruction" of Hgb was thus accelerated.

The spectrum of Methgb (fig. 13, curve VI) changed on the addition of  $\text{H}_2\text{O}_2$ ,  $R = 500$ , within 2 min. to an

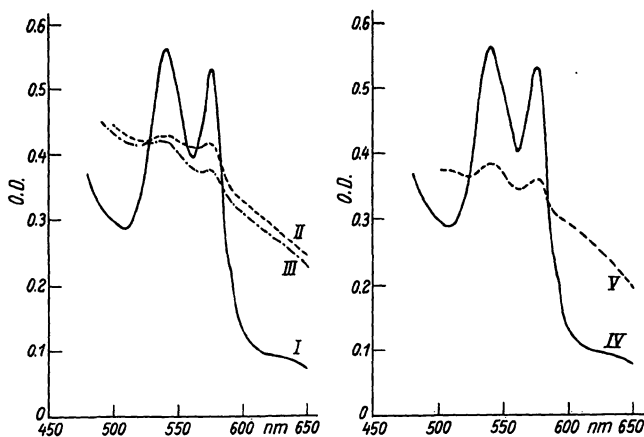


Fig. 11

Spectra of Hgb with and without  $\text{H}_2\text{O}_2$  in tartrate buffer pH 4.2.

Heme = 41.4  $\mu\text{M}$ .  
I = before addition of  $\text{H}_2\text{O}_2$ .  
II = 3 min. after  $\text{H}_2\text{O}_2$ , R = 500.  
III = 15 min. after  $\text{H}_2\text{O}_2$ , R = 500.  
For peroxidase activities see tab. 5

Fig. 12

Spectra of  $\text{HgbO}_2$ : Hp with and with  $\text{H}_2\text{O}_2$ , tartrate buffer pH 4.2.

Heme = 41.4  $\mu\text{M}$ .  
IV = before addition of  $\text{H}_2\text{O}_2$ .  
V = 12 min. after addition of  $\text{H}_2\text{O}_2$ , R = 500. For peroxidase activities see tab. 5

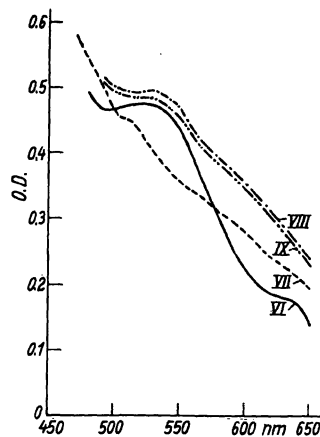


Fig. 13

Spectra of Methgb without and with  $\text{H}_2\text{O}_2$ , tartrate buffer pH 4.2.

Heme = 43.9  $\mu\text{M}$ .  
VI = before addition of  $\text{H}_2\text{O}_2$ .  
VII = 1 min. after  $\text{H}_2\text{O}_2$ , R = 500.  
VIII = 1 min. after  $\text{H}_2\text{O}_2$ , R = 5.  
IX = 12 min. after  $\text{H}_2\text{O}_2$ , R = 5.  
For peroxidase activities see tab. 5

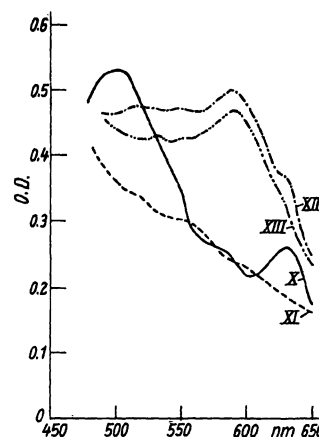


Fig. 14

Spectra of Methgb: Hp with and with  $\text{H}_2\text{O}_2$ , tartrate buffer pH 4.2.

Heme = 43.9  $\mu\text{M}$ .  
X = before addition of  $\text{H}_2\text{O}_2$ .  
XI = 1 min. after  $\text{H}_2\text{O}_2$ , R = 500.  
XII = 1 min. after  $\text{H}_2\text{O}_2$ , R = 5.  
XIII = 12 min. after  $\text{H}_2\text{O}_2$ , R = 5. For peroxidase activities see tab. 5

almost straight declining curve (VII) between 500 and 650 nm.

The activity was only 20% of that before the addition of  $\text{H}_2\text{O}_2$ . On addition of much less  $\text{H}_2\text{O}_2$ , R = 5, the shoulder between 500 and 530 nm rose and the flat peak at 630 nm disappeared already after 1 min. (curve VIII). The activity remained at 100%. This curve could represent the Methgb-OOH complex. After 12 min. the curve (IX) was slightly lower, the activity was still 96%.

The spectrum of Methgb: Hp (fig. 14, curve X) was different from that of Methgb, a peak at 580 nm became visible, the peak at 625 nm shifted to 630 nm and became very pronounced. One min. after the addition of  $\text{H}_2\text{O}_2$  R = 500, the curve (XI) was comparable to the corresponding curve of Methgb (VIII), the activity was diminished to 9%. With  $\text{H}_2\text{O}_2$ , R = 5, (curve XII) the peak at 580 nm shifted to 590 nm after 1 min. and became the maximum of the curve. The activity was 86%. After 12 min. the curve (XIII) was lower and a peak appeared at 530 nm. The activity was 49%. As in the case of Hgb, Hp definitely accelerated the "destruction" of Methgb.

The spectrum of Methgb: Hp in tartrate buffer, pH 4.2 was very similar to the spectrum reported by KEILIN and HARTREE (9) for Methgb in phosphate buffer pH 6.8. We, therefore, recorded the spectra of Methgb and Methgb: Hp under these conditions (fig. 15) and found them to be identical spectra at pH 6.8. Methgb is known to dissociate to hematin and globin at pH < 5. Hp apparently prevents the dissociation and the spectrum of Methgb: Hp was the same or at least very similar to the spectrum of Methgb at pH > 5.

In the presence of  $\text{H}_2\text{O}_2$ , R = 5, the spectrum of Methgb: Hp (fig. 14) had a maximum at 590 nm and resembled the description given by KEILIN and HARTREE

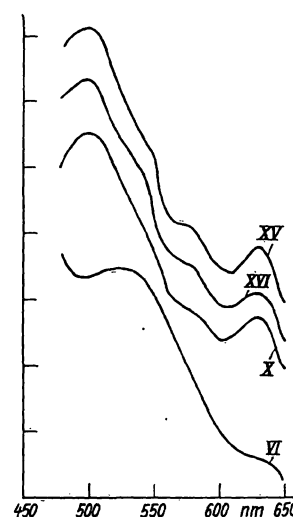


Fig. 15

Spectra of Methgb and Methgb: Hp, in tartrate buffer pH 4.2 and phosphate buffer pH 6.8.

XIV Methgb at pH 6.8. XV Methgb: Hp at pH 6.8. XVI Methgb at pH 4.2 (fig. 13). XVII Methgb: Hp at pH 4.2 (fig. 14). The curves have been displaced vertically for better inspection. Curves X, XIV, and XV are almost identical in absolute values

for Methgb with  $\text{H}_2\text{O}_2$ , R = 100. The compound in our experiment still had 49% of the peroxidase activity of Methgb: Hp.

We do not know yet if any of these spectra are characteristic for the complex of Methgb: Hp with  $\text{H}_2\text{O}_2$ . The only conclusion justified is that Methgb: Hp is much more susceptible to the changes which are brought about with free Methgb with much higher concentrations of  $\text{H}_2\text{O}_2$ . This most likely includes both the formation of the complex with  $\text{H}_2\text{O}_2$  and the destruction of the enzyme.

JAYLE (7) was the first to recognize that the enzymatic characteristics of Hgb: Hp resembled the characteristics of a true peroxidase better than did free Hgb. ROBERT



and SERPICELLI (10) attributed the effect of Hp to the stabilization of Hgb, but the investigations of MORETTI and YON (11) showed unequivocally activation of the peroxidase activity of Hgb by complex formation with Hp. Our data provide evidence that Hp does not stabilize Hgb, but renders Hgb more susceptible to the effects of  $H_2O_2$ , both to the formation of the active complex with  $H_2O_2$  and to the destruction by excess  $H_2O_2$ .

The results of the spectra together with the time reaction curves of the peroxidase activity measurement suggest the following mechanism: the oxidation of Hgb to Methgb and the formation of the Methgb- $H_2O_2$ -complex is faster with Hgb : Hp than with free Hgb, but at the same time the destruction of the enzyme is accelerated when present as the Hp-complex. The curves in figure 3 can be interpreted in this manner:

16 mM  $H_2O_2$ : the Hgb : Hp curve had a much faster initial velocity and a higher max. O. D. than the Hgb curve, because of the increased velocity of the oxidation to Methgb and the formation of the complex with  $H_2O_2$  in the presence of Hp.

128 mM  $H_2O_2$ : the initial reaction of Hgb : Hp was still faster than that of free Hgb, but the destruction of the enzyme was also accelerated so that the curve bends sharply and the max. O. D. was lower than that of the curve with free Hgb. In the latter reaction, the formation of the  $H_2O_2$  complex was faster over a longer period of time than the destruction.

90 mM  $H_2O_2$ : the max. O. D. was equal with both free Hgb and Hgb : Hp, but the initial velocity of the latter was faster and max. O. D. was reached after 3–4 min. as compared to 7–8 min. with free Hgb. Finally the decline of the Hgb : Hp curve was faster than that of the Hgb curve because of the faster destruction of Hgb : Hp.

There was, of course, one fundamental difference in the experimental conditions between the spectra-experiments and the activity measurements. While in the former the ratio of  $H_2O_2$  to the prosthetic groups was between 1 and 500, it was of the order of  $1 \times 10^5$  in the latter. Why then is Hgb not completely inactivated on the addition of  $H_2O_2$ ? We have found that when  $H_2O_2$  was added to Hgb before the chromogen, no color development occurred. The chromogen as such must, therefore, have a protecting influence on the enzyme. With o-tolidine as chromogen a blue oxidation product is formed by peroxidases in the presence of hydroperoxides. The blue compound with an absorption maximum at 630 nm (tartrate buffer pH 4.2) was unstable under our experimental conditions with  $H_2O_2$ . After max. O. D. had been reached, the color was stable for about 8 min. and then faded and turned into a greenish yellow. With Hgb : Hp as enzyme, the blue color was even less stable and we believed for a while that Hp accelerated the oxidation of the blue first product to the second oxidation compound. But the following experiment proved that the second step of conversion is independent of a peroxidase reaction. At the point of maximum color development catalase was added to one of two parallel tests to destroy

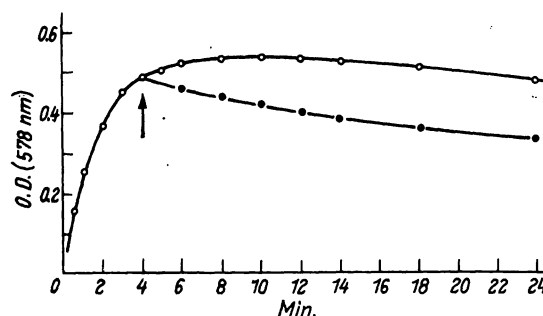


Fig. 17

Effect of catalase on the fading of the blue oxidation product of o-tolidine. The arrow indicates the time, when catalase was added.

excess  $H_2O_2$ . Figure 17 demonstrates that the fading of the blue color was accelerated after the destruction of  $H_2O_2$ .

The catalase experiments suggest the following interpretations:

1. It can be concluded for certain that the oxidation of the blue oxidation product of o-tolidine is independent of  $H_2O_2$ .
2. One can conclude that the oxidation is non enzymatic because it occurs in the absence of the substrate ( $H_2O_2$ ) of the enzyme (Hgb) unless a direct reaction between the chromogen and Hgb without an oxygen donor is postulated, but for which there is no evidence.
3. The faster development of the second oxidation product after  $H_2O_2$  destruction suggests two alternative possibilities:
  - a) Oxygen, developed by the catalase is the oxidizing agent.
  - b) The slower decline of the reaction curve in the presence of  $H_2O_2$  is caused by a continued production of the first oxidation product and an equilibrium exists at the peak of the curve between formation and further oxidation of the blue product. It should be recalled here that Hgb has some catalase effect, so that oxygen is produced from  $H_2O_2$  for the second step of oxidation.
  - c) A combined effect of a) and b).

The diminished stability of the blue oxidation product in the presence of Hgb : Hp is most likely due to the accelerated destruction of the enzyme, which leads to a shortened period of equilibrium between the enzymatic formation of the blue oxidation product and its further non enzymatic oxidation.

While most of the phenomena associated with the peroxidase like activity of Hgb and the Hgb-Hp complex as discussed in the first paper have been dealt with here, we have not described further the effects of sodium alginate. A third paper in this series on this effect and on the nature of the blue oxidation product is being prepared.

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## Zur Bestimmung der „ $\alpha$ -Hydroxybutyratdehydrogenaseaktivität“ im Serum

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Durch methodische und klinische Untersuchungen an 117 Probanden wird gezeigt, daß die Bestimmung der LDH-Aktivität im Serum mit  $\alpha$ -Ketobutyrat bei optimalen Substratkonzentrationen der mit Pyruvat entspricht. Hierdurch wird eindeutig bestätigt, daß die HBDH-Aktivität im Serum der Wirkung der LDH zuzuschreiben ist.

By methodical and clinical studies on 117 probands, it was shown that values for LDH activity, determined in serum with optimal concentrations of  $\alpha$ -ketobutyrate, corresponded to those determined with pyruvate. This confirms that the HBDH activity in serum can be attributed to the action of LDH.

Lactatdehydrogenase<sup>1)</sup> besteht bekanntlich aus vier Monomeren. Durch Kombination der Untereinheiten H und M<sup>2)</sup> entstehen die LDH<sup>3)</sup>-Isoenzyme LDH<sub>1</sub> bis LDH<sub>5</sub>, der Zusammensetzung H<sub>4</sub>, H<sub>3</sub>M, H<sub>2</sub>M<sub>2</sub>, HM<sub>3</sub> und M<sub>4</sub>, die in den einzelnen Organen in unterschiedlichen relativen Mengeneinheiten gebildet werden. Ein weiteres Isoenzym befindet sich in Hoden und Sperma (1).

Da sich Erhöhungen der LDH-Aktivität im Serum bei einer Vielzahl von Krankheitsbildern finden, besitzen Bestimmungen der einzelnen Isoenzyme für die Diagnostik großes Interesse. Eine Vermehrung von LDH<sub>5</sub> im Serum ist für Lebererkrankungen, von LDH<sub>1</sub> und LDH<sub>2</sub> für Herzinfarkt typisch (2).

Die Hydrierung von 2-Ketobutyrat durch Serum und NADH wird häufig von der LDH-Wirkung abgegrenzt und auf eine „ $\alpha$ -Hydroxybutyratdehydrogenase“ zurückgeführt, deren Aktivität kolorimetrisch (3)

oder mit Hilfe des optischen Testes (4) bestimmt wird. Die Bestimmung der HBDH-Aktivität im Serum wird besonders zur Diagnose und Verlaufskontrolle des Herzinfarktes empfohlen, Erhöhungen werden jedoch auch bei Lebererkrankungen, Muskelerkrankungen, Leukämien, megaloblastischen Anämien, nephrotischem Syndrom, Lungenerkrankungen, Tumoren und anderen Erkrankungen beobachtet (5, 6, 7, 8).

Vorliegende Arbeit soll zur Methodik und klinischen Bedeutung dieses Enzymtestes einen Beitrag liefern.

### Methodik

Die LDH-Aktivität wurde nach WROBLEWSKI und LADUE (9, 10) mit Hilfe des optischen Tests bei 340 nm im 3 ml Testansatz bestimmt. Die Pyruvatkonzentration betrug 0,3 mM. Die Messung der HBDH-Aktivität erfolgte analog mit Natrium- $\alpha$ -ketobutyrat, das aus  $\alpha$ -Ketobuttersäure hergestellt wurde.

Mit verschiedenen Konzentrationen an  $\alpha$ -Ketobutyrat wurde die optimale Substratkonzentration zur Aktivitätsbestimmung der HBDH in Normals Serum, Serum nach Herzinfarkt und bei Hepatitis ermittelt und bei den folgenden Messungen angewandt.

Die NADH-Lösung wurde jede Woche frisch hergestellt (11,12). Das Serum der Blutproben wurde sofort gewonnen, da sonst Erhöhungen der Fermentaktivität auftreten (13). Die Standardabweichung für die LDH- und HBDH-Aktivität wurde aus 10 Bestimmungen an einem Serum zu  $s = \pm 4,8$  I. U. ermittelt.

Das Verhalten verschiedener Inhibitoren auf die LDH- und HBDH-Aktivität wurde von uns vergleichend untersucht.

Sulfitionen zeigen besonders große Inhibitorwirkung auf die LDH. Das Herzmuskelisozym wird durch Bindung von Hydro-pyridin-4-sulfonsäure am stärksten gehemmt (14). Oxalat besitzt eine ähnliche Wirkung (15).

<sup>1)</sup> Der Trivialname Lactatdehydrogenase wird hier gebraucht für das Enzym L-Lactat: NAD-Oxydoreduktase (EC1.1.1.27), Glutamat-Pyruvat-Transaminase für L-Alanin: 2-Oxoglutarat Amino transferase (EC 2.6.1.2), Glutamat-Oxalacetat-Transaminase für L-Aspartat: 2-Oxoglutarat Aminotransferase (EC 2.6.1.1).

<sup>2)</sup> Nach Cahn und Mitarbeiter wird die schnellste anodische Fraktion (H<sub>4</sub>) als LDH<sub>1</sub>, die kathodische Fraktion (M<sub>4</sub>) als LDH<sub>5</sub> bezeichnet.

<sup>3)</sup> Abkürzungen:

LDH = Lactatdehydrogenase, GOT = Glutamat-Oxalacetat-Transaminase, GPT = Glutamat-Pyruvat-Transaminase, HBDH =  $\alpha$ -Hydroxybutyratdehydrogenase. I. U. = Internationale Einheiten.